

Left ventricular wall stress as a direct correlate of cardiomyocyte apoptosis in patients with severe dilated cardiomyopathy

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Background Apoptosis has been implicated as a possible mechanism in the development of heart failure (HF), but the mechanisms involved remain unclear. In patients with severe dilated cardiomyopathy, we evaluated cardiomyocyte apoptosis in relation to the transmural distribution of Bax and Bcl-2 proteins (2 molecules inhibiting or promoting apoptosis, respectively) and left ventricular wall stresses.

Methods We studied the presence and distribution of cardiomyocyte apoptosis in 90 tissue samples obtained from 8 patients who were undergoing left ventricular reduction with the Batista (ventricular remodeling) operation. Apoptosis was assessed in tissue samples taken from the entire left ventricular thickness (subdivided in subepicardial, midmyocardial, and subendocardial sections) with the terminal deoxynucleotidyl transferase mediated dUTP-biotin nick-end labeling (TUNEL) technique and DNA agarose gel electrophoresis. The expression of Bcl-2 and Bax proteins were determined with both Western analysis and immunohistochemistry.

Results TUNEL-positive cells (apoptotic index) were $2.3\% \pm 1.4\%$. Apoptotic cells were predominantly distributed in the subendocardium, where higher levels of Bax protein were detected. The ratio of Bax to Bcl-2 proteins (Bax/Bcl-2) was similar in the midmyocardium or subepicardium, but increased in the subendocardium, where it was directly related to systolic wall stress ($y = 0.009x - 0.629$; $r^2 = 0.85$, $P < .001$). The apoptotic index was also directly related to systolic and end-diastolic stresses calculated from hemodynamic and echocardiographic data ($r^2 = 0.77$, $P < .001$ and $r^2 = 0.40$, $P < .01$, respectively).

Conclusions In patients with dilated cardiomyopathy, in whom cardiomyocyte apoptosis is an important cause of cell loss, apoptosis is more extensively localized in the subendocardium and strictly related to ventricular wall stresses and the Bax/Bcl-2 ratio. (Am Heart J 2003;146:1105–11.)

Apoptosis (programmed cell death) is a potentially important cause of cell loss in several cardiac pathological conditions, including acute myocardial infar-

tion,¹ ischemia-reperfusion damage,² and cardiomyopathies.^{3,4} The loss of cardiomyocytes in dilated cardiomyopathy has been linked to the progressive decline in left ventricular function and congestive heart failure.⁵ Recent studies have proposed that myocyte loss by apoptosis can occur as the consequence of prolonged growth stimulation of adult, terminally differentiated myocytes.⁶ To meet chronically altered hemodynamic demands in the failing myocardium, growth stimulation would initially occur as a compensatory mechanism, likely mediated by the up-regulation of systemic or local mediators of the adrenergic⁷ or the renin-angiotensin systems⁸ and by various cytokines, such as tumor necrosis factor- α (TNF- α).⁹ These factors induce the expression of immediate-early genes, which in turn lead to increased protein synthesis and myocardial hypertrophy, but also modulate the

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Table I. Clinical characteristics, echocardiographic and hemodynamic measurements of the study patients (n = 8)

Age (y)	(range 57–75)
Sex (m/f)	6/2
NYHA class III/IV	5/3
Diuretics	8
ACE inhibitors	8
IV inotropes	1
Digoxin	5
Nitrates	6
β -Blockers	3
Main echocardiographic data	
Left ventricular volume (mL/m ²)	
End-systolic	177 \pm 51
End-diastolic	213 \pm 94
Ejection fraction (%)	21 \pm 12
Main hemodynamic data	
Cardiac index (mL/min/m ²)	2120 \pm 344
Mean pulmonary-artery wedge pressure (mm Hg)	28 \pm 8
Right ventricular end-diastolic pressure (mm Hg)	13 \pm 6

Data are mean \pm SD. ACE, Angiotensin converting enzyme; NYHA, New York Heart Association; IV, intravenous.

expression of pro-apoptotic (such as p53, Bax) and anti-apoptotic (Bcl-2) proteins.^{10–12} Mechanisms implicated in apoptosis in the failing human heart have, however, remained elusive. Inflammatory cytokines,¹³ reactive oxygen species,¹⁴ nitric oxide,¹⁵ hypoxia,^{16,17} reperfusion,¹⁸ and growth factors¹⁹ have all been pathogenetically implicated. A role of mechanical stretching and wall stresses has been recently hypothesized.^{20–22} However, this mechanism has been sufficiently demonstrated only in the isolated rat papillary muscle.²³ We postulated the relevance of this mechanism in the progression of heart failure in humans, as part of a vicious circle begetting heart failure through the induction of apoptosis. We therefore determined apoptosis, with some of its molecular correlates, in tissue samples obtained in humans with chronic heart failure undergoing left ventricular reduction with the Batista operation, yielding an unique opportunity for the immediate access to fresh human myocardial tissue for the study of apoptosis in the failing heart.

Methods

Patients

The study group consisted of 8 consecutive patients (from January 1997 to May 1998) with dilated cardiomyopathy (6 cases caused by valvular disease and 2 cases caused by ischemic heart disease) who were undergoing the Batista operation. Details of the patients recruited are given in Table I. To confirm the diagnosis, all patients underwent a full cardiologic examination, including medical history and physical examination, laboratory investigations, M-mode, 2-dimensional and Doppler echocardiography, and cardiac catheterization and coronary arteriography. On the basis of hemodynamic

(aortic and left ventricular pressure) and echocardiographic data (systolic and end-diastolic diameters, left ventricular mass), end-diastolic and systolic wall stresses (WS) were calculated. These were obtained by using the Laplace formula²⁴:

$$WS = P \times 2r/4W \quad (1)$$

in which WS is systolic or end-diastolic wall stress (kdynes/cm²), P is systolic blood pressure or left ventricular end-diastolic pressure, 2r is end-systolic or end-diastolic left ventricular diameter, and W is end-systolic or end-diastolic left ventricular wall thickness. All patients were in New York Heart Association class III or IV; their preoperative mean ejection fraction (EF) was 21% \pm 12%. All patients underwent left ventricular reduction with the Batista operation (or wedge resection of the heart), a surgical technique used in recent years (but currently abandoned in our center), as a bridge to heart transplantation to reduce ventricular volumes and improve ventricular performance by restoring better chamber diameters and size.^{25,26} This technique consists of a longitudinal section of the left ventricular free wall, with resection of a piece of ventricle and, sometimes, the replacement of mitral valve to avoid valve insufficiency.

Sample selection and collection

In the selection of myocardial tissue for excision, resections were performed distal to the site of any myocardial infarction and avoiding tissue appearing fibrotic or necrotic. The excised tissue was longitudinally sectioned from base to apex (2–3 mm/slice). Overall, 90 tissue samples were examined (n = 12 for each patient). Four transmural myocardial subspecimens were derived at random from each original surgical specimen. We subdivided each myocardial subspecimen into 3 layers of approximate equal thickness (subepicardial, midmyocardial, and subendocardial), to derive 90 samples in the 8 patients studied. Tissue sections were immediately processed, fixed in 10% buffered formalin, or frozen in liquid nitrogen and stored at -80°C .

Agarose DNA gel electrophoresis

Freshly isolated myocardium (200–500 mg) was minced in an equal volume of homogenization buffer (10 mmol/L Tris-HCl, 25 mmol/L EDTA, and 100 mmol/L NaCl; pH, 8.0) at 4°C , and homogenized for 30 seconds with a Polytron homogenizer at 10,000 rpm. A 100 μL aliquot of the homogenate was then mixed with 1.25 mL of lysis buffer (10 mmol/L Tris-HCl, 25 mmol/L EDTA, 100 mmol/L NaCl, and 1.0% sodium-dodecyl-sulphate [SDS]; pH, 8.0), and the suspension was subsequently centrifuged at $13,000 \times g$ for 15 minutes at room temperature. The supernatant, which is enriched in soluble fragmented DNA, was poured off and collected, taking care to leave the viscous pellet containing the intact DNA behind. The supernatant was treated with proteinase K (100 $\mu\text{g}/\text{mL}$, Sigma, Milan, D) for 30 minutes at 50°C . Ethanol (final concentration, 60%) and NaCl (final concentration, 0.5 mol/L) were then added, and the DNA was precipitated overnight at -20°C . The DNA was collected by centrifugation at $13,000 \times g$ for 15 minutes at 4°C , dissolved in 500 μL of TE buffer (10 mmol/L TRIS-HCl and 1 mmol/L EDTA; pH, 8.0), and extracted once with phenol/chloroform saturated with TE buffer. The DNA solution was washed once with chloroform and precipitated in 60% ethanol on 0.5 mol/L NaCl at

-20°C for 1 hour. The DNA collected with centrifugation was dried, dissolved into 50 µL TE buffer, treated with RNase (100 µg/mL, Sigma) for 30 minutes at 37°C, and then immediately subjected to electrophoresis on agarose gels (1.5%) in TAE buffer (40 mmol/L Tris-HCl, 30 mmol/L acetic acid, and 2 mmol/L EDTA; pH, 8.0).

Terminal deoxynucleotidyl transferase-mediated dUTP-biotin nick-end labelling

Apoptosis was detected in situ on left ventricular cross sections with terminal deoxynucleotidyl transferase-mediated dUTP-biotin nick-end labelling (TUNEL). Cross sections were dewaxed, rehydrated, and incubated in 20 µg/mL proteinase K (Sigma) for 1 hour. Endogenous peroxidase was blocked with incubation in 3% hydrogen peroxide for 5 minutes. Fragmented DNA were nick end-labeled with a mixture of terminal deoxynucleotidyl transferase (TdT, 22.5 U/section; Boehringer Mannheim, Mannheim, D), and biotinylated dUTP (0.2 mmol/section) in a TdT buffer (30 mmol/L Tris-HCl, 140 mmol/L sodium cacodylate, 1 mmol/L cobalt chloride) for 90 minutes at 37°C. The reaction was stopped with incubation for 15 minutes in 0.5 mol/L EDTA. The biotinylated fragmented DNA were detected with 25 minutes of incubation in streptavidin-conjugated peroxidase, followed by 15 minutes of incubation in aminoethyl carbazole. Sections were counterstained with Mayer's hematoxylin. Cells with a brown-red nuclear labeling were defined as TUNEL-positive. Positive controls were provided by sections pretreated with DNase I (100 U/mL; Boehringer Mannheim) that demonstrated staining of all nuclei. In negative-control experiments, TdT was omitted from the labeling mixture. The entire stained section was scanned at 100× using light microscopy. When TUNEL-positive cardiomyocytes were detected, the magnification was changed to 400× to assess whether staining occurred in cardiomyocytes or non-cardiomyocytes. Only TUNEL-positive cardiomyocytes were counted in each slide. We classified cardiomyocytes as TUNEL-positive when stained nuclei were inside the cardiomyocyte contour. Any nuclei that were ambiguous were not counted; also, round homogeneous dark dots (considered to be artifacts) and stained cell debris were carefully eliminated from counting. An apoptotic index was calculated as the percentage of TUNEL-positive cell nuclei (number of TUNEL-positive cell nuclei + number of total cell nuclei).

Immunohistochemistry

Frozen section (5–6 µm) were stained with PBS, with a murine irrelevant primary antibody, and with rabbit serum as negative controls, and with alpha-sarcomeric actin as a positive control. Antibodies against Bax and Bcl-2 proteins (Dako, Cambridgeshire, UK) were incubated on serial sections with a second layer of biotinylated porcine anti-rabbit immunoglobulins (Dako), followed by streptavidin-peroxidase complex (Dako). Reactivity was detected with diaminobenzidine tetrahydrochloride (25 mg/mL) and hydrogen peroxide (0.01% w/v). Sections were counterstained with Mayer's hematoxylin. The reactivity of the different proteins was semi-quantitatively graded according to their intensity within the entire section. Densitometric analysis of Bax and Bcl-2 proteins was performed with a Sony video camera connected to

a Leica Quantimet 500 plus (Leica Cambridge, Cambridge, UK), determining the change in integrated optical density (IOD).²⁷

Western analysis of Bcl-2 and Bax proteins

Tissue samples (100 mg) from each anatomical zone were homogenized with 500 µL lysis buffer (1% Nonidet P-40, 0.5% sodium deoxycholate: 0.1% SDS in 1× PBS) at 4°C for 20 seconds, incubated on ice for 2 to 3 hours, and then centrifuged twice at 16,000 × g for 20 minutes. Protein concentration was measured with the DC Protein Assay (Bio Rad, Hercules, Calif). An 80 µg amount of total protein for each lane was mixed with loading buffer (5% beta mercaptoethanol, 0.05% bromophenol blue, 75 mmol/L Tris-HCl; pH, 6.8; 2% SDS and 10% glycerol), boiled for 4 minutes, and loaded onto a 4% to 15% gradient SDS-polyacrylamide gel using a Mini Protean II Dual Stab Cell (Bio Rad). Proteins were transferred on nitro-cellulose filter in the presence of a glycine/methanol transfer buffer (20 mmol/L Tris base, 0.15 mol/L glycine, 20% methanol) in Mini Protean Transfer (Bio Rad). The nitrocellulose filter was blocked with 6% milk in 1× TBS-T buffer (20 mmol/L Tris-HCl; pH, 7.6; 137 mmol/L NaCl, 0.05% Tween-20) for 1 hour at room temperature. Membranes were subsequently exposed to rabbit polyclonal anti-rat Bcl-2 and rabbit polyclonal anti-rat Bax (Pharmingen, San Diego, Calif), respectively, at 1:1000 concentration in 6% milk in TBS-T for 1 hour. Bound antibody was detected with horseradish peroxidase-conjugated anti-rabbit immunoglobulin-G. Finally, enhanced chemiluminescence detection reagents were used as a means of visualizing peroxidase reaction products (Bio Rad). Bcl-2 protein was detected as a 29-kDa band, and Bax protein as a 26-kDa band, using molecular weight markers as reference.

Statistical analysis

All tissue samples were coded, and the code was only broken at the end of the studies. Results are presented as the mean plus or minus SD. Statistical significance ($P < .05$) was determined with the 2-tailed Student *t* test (paired or unpaired as appropriate) for single comparisons and with analysis of variance (ANOVA) for multiple comparisons, respectively, after checking for the normality of distributions. Linear regression analysis was performed to test for correlation between 2 variables.

Results

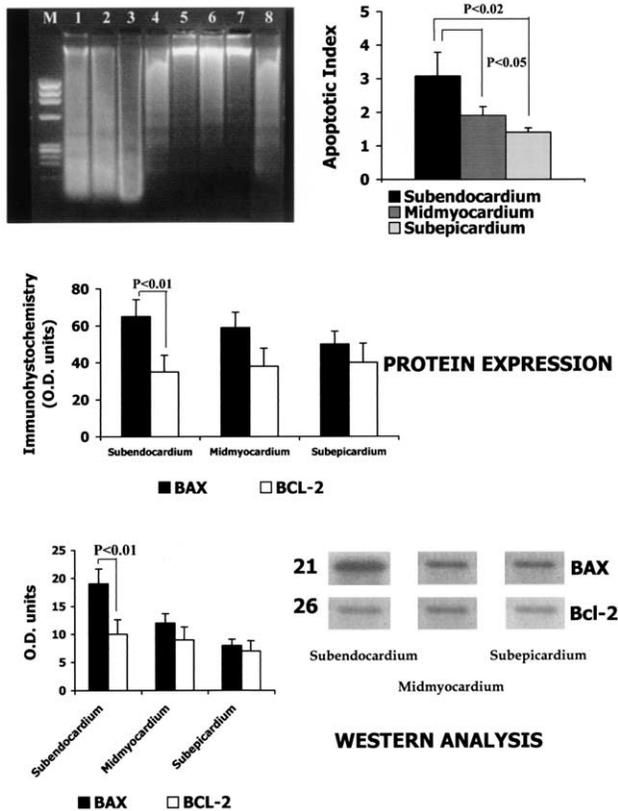
DNA fragmentation

There was a very faint presence of DNA ladders in samples provided from all patients (Figure 1, top left panel). In some samples there was a light degree of smearing, suggestive of cellular necrosis.

TUNEL

In all negative controls, in which TdT was omitted, no positive reactivity was detected. Sections treated with DNase 1 as a positive control showed positive staining of all the nuclei. Tdt-mediated dUTP nick end-labeling positivity was predominantly confined to myo-

Figure 1

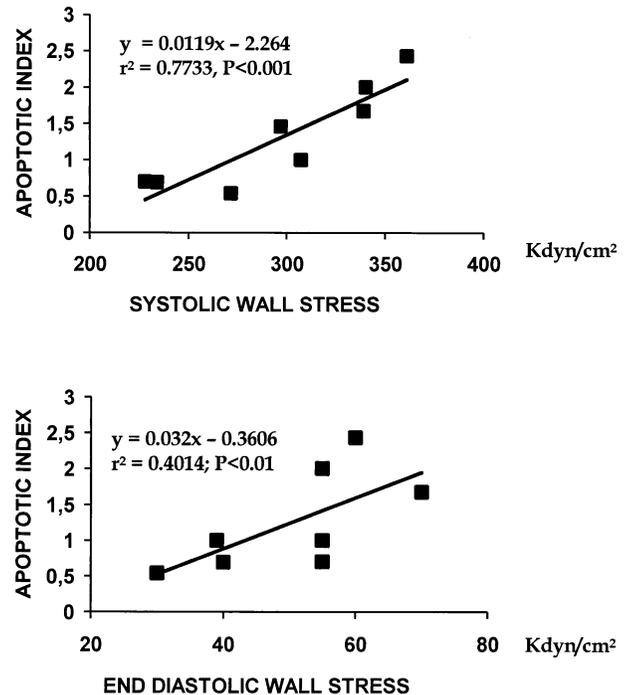


Top left panel: ethidium bromide staining of genomic DNA extractions in myocardial tissue samples from the 8 patients (numbered 1 to 8), after electrophoretic separation in 1.5% agarose gels. Lane 1: M = DNA molecular weight markers (kbp). Top right panel: distribution of the apoptotic index (% of apoptotic cells, as detected with the TUNEL technique, \pm SD) from all patients in the subendocardium, midmyocardium, and subepicardium. Middle panel: densitometric data, in arbitrary units of optical density \pm SD, from immunohistochemical determination of Bax and Bcl-2 proteins in tissue sections (15–20/patient) from all patients. Bax intensity was significantly higher in the subendocardium than the subepicardial and midmyocardial samples. Bcl-2 staining was generally weaker, with no significant transmural differences in staining intensity. Bottom panel: Western blot analysis, in arbitrary units of optical density \pm SD, of Bax and Bcl-2 expression in myocardial samples with blot densitometric quantitative analysis in all patients is shown on the left, and 1 representative experiment from 1 patient is shown on the right.

cytes, although occasional endothelial cells were seen as TUNEL-positive within some sections from all patients.

The percentage of TUNEL-positive cells (apoptotic index) was 2.3 ± 1.4 . Apoptotic cells were not seen uniformly across sections, but were randomly scattered

Figure 2



Linear regression analysis and Spearman correlation coefficients between systolic (top) or end-diastolic (bottom) wall stresses and the apoptotic index.

and predominantly distributed in the subendocardium (Figure 1, top right panel). The apoptotic index was strictly and directly related to systolic and end-diastolic stresses, calculated from hemodynamic and echocardiographic data (end-diastolic wall stress: $y = 0.0119x - 2.264; r^2 = 0.77; P < .001$; systolic wall stress: $y = 0.032x - 0.3606; r^2 = 0.40; P < .01$; Figure 2).

Immunohistochemistry

The pattern of distribution of Bax and Bcl-2 proteins was similar in all patients. Bax intensity was significantly higher in the subendocardium than the subepicardium and midmyocardium. Bcl-2 staining was generally weak, uniform, and localized within myocytes in all samples analyzed. No significant differences in Bcl-2 staining intensity were detected in the subendocardial, midmyocardial, or subepicardial samples (Figure 1, middle panel).

Western analysis for Bax and Bcl-2 proteins

Bcl-2 staining was generally weak and uniformly localized, with no significant differences in staining intensity in subendocardial, midmyocardial or subepicardial samples. On the contrary, a subendocardial-subepicardial gradient was detected for Bax protein,

with higher levels in the subendocardium. Bax/Bcl-2 was similar in subepicardial and midmyocardial samples and increased in the subendocardium (Figure 1, bottom panel), where it was directly related to systolic wall stress ($y = 0.009x - 0.629$; $r^2 = 0.85$, $P < .001$), but not significantly to end-diastolic wall stress ($y = 0.139x + 9.258$; $r^2 = 0.28$, $P =$ not significant)

Discussion

This is the first study demonstrating that apoptotic cell death is strictly related to left ventricular wall stresses and mostly localized in the subendocardium in patients with severe dilated cardiomyopathy. The unique opportunity provided by the surgical setting and the absence of delay from tissue excision to processing unequivocally confirmed, in humans, the presence and extent of apoptosis, consistent with a pathophysiological role for the balance of the pro-apoptotic Bax and the anti-apoptotic Bcl-2 proteins.¹²

There are important methodological limitations in assessing apoptosis in human heart failure. The exiguity of apoptotic cells and their spatially variable distribution make the quantitative assessment of apoptosis difficult.³ Of the various methods for detecting apoptosis, so far only a few have been applied to investigate apoptosis in human heart failure. In general, the TUNEL method has been the most frequently used. This method has the main advantage of allowing the *in situ* detection and identification of cells with DNA fragmentation. Despite controversy on specificity and sensitivity, it can provide a valid quantification of apoptosis when associated with other techniques.^{1,10} These were, in our study, the detection of DNA laddering and the expression of the apoptosis-regulating proteins Bax and Bcl-2. Studies on human myocardial apoptosis have been so far performed in tissue samples obtained from patients after cardiac transplantation or post-mortem,^{3,4,11,28} with 1 more recent study performed on endomyocardial biopsies.²⁹ Limits of these studies are the delay (various hours) elapsing from patient's death to the analysis (in all previous studies) or the limited amount of tissue samples available.²⁹ For these reasons, the quantification of apoptosis and investigations on the mechanisms involved in relation to the progression of heart failure have remained controversial.¹⁰ We exploited the unique opportunity for the immediate access to fresh human myocardial tissue offered by surgical interventions of left ventricular reduction with the Batista operation.^{25,26} Apoptosis was documented histologically with the TUNEL technique and biochemically with DNA agarose-gel electrophoresis. These methods identify double-strand cleavage of the DNA in myocyte nuclei and DNA laddering in the myocardium, respectively. The extent of TUNEL-positive cardiomyocytes was found $2.3\% \pm 1.4\%$, which is in agreement

with most recent literature, in which the apoptotic index in congestive heart failure has been reported to range from 0.2% to 30%.^{10,28,29} The chronicity of the apoptotic process and the relevant percentage of TUNEL-positive cells detected in our patients would imply a considerable loss of viable myocardial cells in the long term and a likely relevant role in the progressive deterioration of left ventricular function in these patients with advanced heart failure. The percentage of TUNEL-positive cells was twice as high in the subendocardium as the subepicardium and correlated well with the significantly higher subendocardial expression of the apoptotic protein Bax, with Western analysis, compared with the subepicardium.

The presence of DNA laddering was limited or, frequently, absent in our samples. The difficulty in detecting DNA laddering may be explained by the low and variable level of apoptotic cells within heterogeneous pieces of tissue,^{3,28} and the low sensitivity of this method, which is only possibly circumventable through the selective amplification of DNA fragments with ligation-mediated polymerase chain reaction (PCR).³⁰ Significantly higher levels of the pro-apoptotic Bax protein levels were seen in the subendocardium than in other myocardial layers, whereas the levels of the anti-apoptotic Bcl-2 protein were low and uniformly distributed transmurally through the myocardium. The Bax/Bcl-2 ratio was similar in the midmyocardial and subepicardial samples and increased in the subendocardium, where apoptosis was well represented. These results are in partial agreement with data from Latif et al¹¹ and Olivetti et al,³ in which Bax and Bcl-2 protein levels were directly or inversely related to apoptosis, respectively, but were assessed in the whole heart without attempts at identifying transmural gradients. We detected no significant differences in the Bcl-2 protein distribution, which suggests the absence, at least within our samples, of the compensatory anti-apoptotic mechanism that was previously suggested in the whole heart in patients with heart failure compared with control subjects.¹¹

Our study, for the first time in humans, shows a direct correlation between apoptosis and systolic wall stresses, diastolic wall stresses, or both (obtained from easily accessible hemodynamic and echocardiographic data). This observation is supported by the transmural distribution of apoptosis within the left ventricular wall, with apoptosis being significantly higher in the subendocardial samples, in which mechanical stresses are typically higher, than in midmyocardial and subepicardial samples.

Left ventricular chamber enlargement is a characteristic adaptation of the failing heart. This, in turn, is known to induce a significant increase in systolic and diastolic wall stresses,^{10,31,32} which are not reversed by compensatory mechanisms such as myocardial hyper-

trophy. These changes induce an up-regulation of the renin-angiotensin system^{33,34} and an increase in catecholamines^{35,36} and various cytokines, all of which are potentially able to induce apoptosis.²³ A reduction in the expression of the anti-apoptotic insulin-like growth factor-1 might also contribute.²¹ Whatever fine mechanisms are involved, these changes would hesitate into increased apoptosis, possibly leading to further progression of heart failure.

Specific limitations are to be considered in the interpretation of these data. The number of patients studied ($n = 8$) is admittedly limited (such interventions are no longer performed), but our study group is homogeneous. In addition, the number of samples used in histological and molecular assays ($n = 90$) appears adequate. The TUNEL technique has intrinsic limitations caused by an overestimation of apoptotic cells, because it partially detects living cells with increased activity of DNA repair.²⁹ However, this technique provides an easily accessible histochemical method for the investigation of apoptosis, yielding results in excellent agreement with those of other techniques (eg, electron microscopy).³⁷ The presence of the typical DNA laddering and the changes of Bax/Bcl-2 independently support in our study the existence of a pathophysiologically relevant amount of apoptotic cells within the failing myocardium.

Clinical relevance and implications of cardiomyocyte apoptosis in severe dilated cardiomyopathy

Although the significance of cardiomyocyte apoptosis in the pathophysiology of heart failure due to dilated cardiomyopathy currently remains uncertain, we postulate a relevant role of this process in disease progression. The main reason for this is in the extent of this process in our specimens. With an apoptotic cycle as rapid as 6 to 24 hours, the high percentage of TUNEL-positive cells detected in our specimens at a single time point ($2.3\% \pm 1.4\%$), despite all the aforementioned limitations, implies, when sustained, a considerable loss of viable myocardial cells with time. For these reasons, the use of drugs directly preventing apoptosis or exerting protection against apoptotic triggers (ie, increased wall stress, oxidative damage, hypoxia, activation of the renin-angiotensin and the adrenergic systems) such as, potentially, angiotensin-converting enzyme inhibitors,³⁸ angiotensin type-1 receptors blockers,³⁹ β -blockers,⁴⁰ and statins⁴¹ might significantly slow down the progressive loss of cardiac cells and retard the development of end-stage heart failure also through this mechanism.

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